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Lack of effect of anaerobiosis, ethylenediamine tetraacetic acid (EDTA) and ouabain on release or on chlorpromazine-induced inhibition of release of lysosomal acid phosphatase *in vitro**

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CHLORPROMAZINE (CPZ) appears capable of stabilizing the rat liver lysosomal membrane both *in vitro* and *in vivo*[1]. The stabilization was evidenced by an inhibition of "release" of protein and the lysosomal enzyme acid phosphatase. Several mechanisms have been proposed to explain such a membrane stabilization, but none has been examined in detail. The stabilization of a number of cell and organelle membranes including those of the mitochondrion, plasma membrane of nerve cells (synaptosome), lysosome and red blood cell by CPZ is now well documented.[2] Duncan[3] has emphasized the similarities of these membranes. The "release" of acid phosphatase from isolated rat liver lysosomes is used in this report as a model system testing three hypotheses put forth to explain the general stabilization of membranes produced by CPZ. The three hypotheses are: (1) that membranes are subject to labilization by lipid peroxidation and that CPZ prevents the peroxidative labilization, possibly by serving as an alternate substrate; (2) that certain metals induce membrane labilization and that CPZ may interact with these metals; (3) that an adenosine triphosphatase (ATPase) is involved in membrane function and that CPZ may interact with this ATPase.

Desai *et al.*[4] demonstrated that rat liver lysosomes as well as mitochondria and microsomes peroxidized and that there was a release of arylsulfatase from the lysosomes which paralleled the peroxidation. Bernheim[5] reported that CPZ and an "activated" product of CPZ were capable of inhibiting lipid peroxidation as measured by the thiobarbituric acid method.[6]

We have repeated and confirmed the observations of Bernheim[5] that CPZ (1×10^{-4} M) completely inhibited lipid peroxidation in rat liver whole homogenate as well as in mitochondrial and lysosomal fractions of rat liver. We have also confirmed the findings of Desai *et al.*[4] that the lysosomal fraction peroxidizes more slowly than the mitochondrial fraction. CPZ at 5×10^{-5} M and 1×10^{-4} M completely inhibited peroxidation in these fractions as measured by the thiobarbituric acid method. To determine whether the CPZ prevented the peroxidation by serving as an alternate substrate, TLC examination was performed. The incubation mixture was acidified with conc. HCl and heated to 100° for 8 min and then centrifuged. The clear supernatant was adjusted to pH 9 and extracted with ethylene dichloride (10%) in isoamyl alcohol. The organic solvent layer was evaporated to dryness and the residue dissolved in methanol and spotted on Gelman thin-layer medium type A. The chromatograms were run in two solvent systems, chloroform–*n*-propanol (10:1) and *n*-propanol. Color was developed by concentrated sulfuric acid spray. Under these conditions, the incubation extract showed a CPZ spot and a spot different from CPZ, CPZ sulfoxide, and mono- or didesmethyl CPZ. This unidentified spot may be similar to Bernheim's "activated" CPZ or it may be CPZ oxidized to something other than the sulfoxide by the peroxidizing medium. Control experiments demonstrated only CPZ spots when tissue was absent or when incubation time was only 10 min. Further characterization has not been attempted because of the apparent lack of involvement of peroxidation in labilization or stabilization phenomena in lysosomes as outlined below.

In order to determine the importance of peroxidation in release of lysosomal enzymes under our conditions of incubation,[1] the lysosomal fraction of rat liver[1] was incubated anaerobically in a closed vessel after 15 min of bubbling with a 95% nitrogen 5% CO_2 mixture. Four experiments under these conditions (1 hr of incubation, 37°, pH 5·0, 0·6 M sucrose) found a mean "release" of acid phosphatase into the supernatant of 35·7 ± 2·9 per cent of the total acid phosphatase. In four parallel experiments incubating in air, the lysosomes "released" 34·0 ± 1·6 per cent of their total acid phosphatase into the supernatant. Thus, prevention of peroxidation did not alter "release" of lysosomal enzyme. The thiobarbituric acid method for determining lipid peroxidation was applied in two such experiments and provided verification that no peroxidation occurred in the medium previously gassed with N_2–CO_2 and incubated anaerobically, but there was peroxidation in the control medium in air. In four other experiments the lysosomal suspension was bubbled with 95% O_2–5% CO_2 and acid

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phosphatase "release" with and without CPZ was compared to control suspensions in air. Oxygenation produced no noticeable effect on phosphatase release. These results confirm the earlier reports of deDuve and Beaufay[7] and of Sledge and Dingle.[8] CPZ, 5×10^{-4} M, inhibited the "release" of enzyme equally well in oxygenated, nitrogenated and aerated lysosomal suspensions. Spirtes *et al.*[9] reported the absence of lipoperoxidation, as measured by the thiobarbiturate method, in mitochondria undergoing swelling. It should be noted that these mitochondria were in rather dilute suspension.

In order to test the possibility that CPZ stabilization of lysosomal membrane involves interactions with metals, disodium EDTA (1×10^{-3} M) was added to the incubation medium. In two experiments, EDTA did not alter either the "release" of lysosomal acid phosphatase or the inhibition of that release produced by CPZ (5×10^{-4} M).

Likewise, ouabain in concentrations of 1×10^{-4}M, 5×10^{-4}M, 1×10^{-6}M and 1×10^{-8}M had no effect on enzyme "release" or on CPZ (5×10^{-4}M) inhibition of enzyme "release". An ATPase not inhibited by ouabain has been suggested by Duncan[3] to be of importance in the labilization of the lysosomal membrane. Thus, it is not possible to dismiss the importance of an ATPase in lysosomal membrane function. In this regard it may be recalled that CPZ itself inhibits the activity of several ATPases such as the mitochondrial Mg^{2+}-activated ones, but not others.[10] Since divalent cations are required for the activation of certain ATPases, the lack of evidence of interaction of certain ATPases, the lack of evidence of interaction between CPZ and EDTA tends to cast further doubt on the involvement of an ATPase in lysosomal membrane function.

Of the three hypotheses, only the one concerning lipid peroxidation has been examined under sufficiently stringent conditions to warrant its rejection. The evidence concerning the other two hypotheses, although consistently negative, is not sufficient to permit their complete rejection. In our opinion, however, the mechanism of stabilizing action of CPZ on lysosomes must be sought elsewhere such as on biophysical grounds.

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Repair of sub-lethal damage of L5178Y lymphoblasts *in vitro* treated with dimethyl myleran and nitrogen mustard

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IN STUDYING mechanism of cell kill by physical or chemical agents, one may examine either the damaging or the recovery process.[1] With chemicals the damaging process includes such considerations as dose-dependence, membrane permeability, inactivation of drug, and reduction in cytocidal effect